REMARKS

Reconsideration of the above-identified application in view of the following remarks is respectfully requested.

I. Status Of The Claims

Claims 36-58, 61-75, 77 and 84-92 are pending in this application, of which claims 36-58, 61, 64-75 and 77 have been withdrawn from consideration. Claims 62, 63 and 84-92 of the elected Group VII, i.e. the compounds of formula VI), were examined in the November 2, 2005 final office action.

The withdrawn claims of Group I (claims 36 and 67-72) are process claims that include all of the limitations of the elected product claims. The withdrawn claims have been maintained as pending in the application. In accordance with MPEP §821.04 and *In re Ochiai*, 71 F.3d 1565, 37 USPQ 1127 (Fed.Cir.1995) the Examiner is requested to rejoin the withdrawn process claims upon allowance of product claims.

Applicant reserves the right to file one or more divisional or continuation applications directed to any cancelled subject matter or any other subject matter disclosed in the application but not encompassed by the pending claims.

No additional amendments are being made by way of this reply.

II. Rejection Under 35 U.S.C. § 102

Claims 62, 63, and 84-92 have been rejected under 35 U.S.C. 102(b) as allegedly anticipated by Soukchareun et al. (*Bioconjugate Chem.* **1998**, 9, 466-475; "Soukchareun"). The Examiner asserts that Soukchareun teaches the compounds of the elected invention.

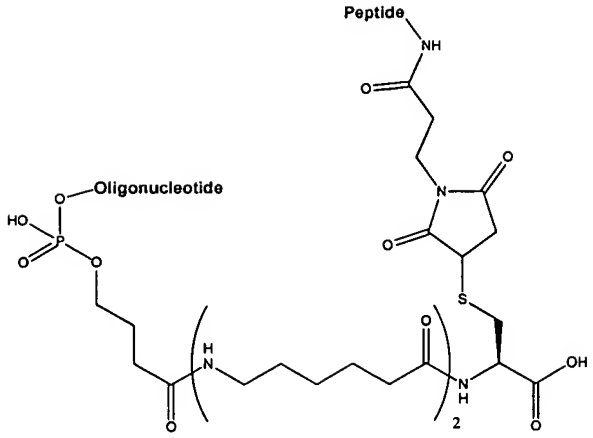
Applicants respectfully submit that it is unclear how the Examiner has reached this conclusion. The Examiner states on page 2 of the Action that "Soukchareun et al. teach the elected

invention, namely a compound of formula IV[1] consisting of peptide residue (cysteine) conjugated to an oligonucleotide [...]." However, the corresponding coupling process disclosed in that document (*See* Soukchareun, Scheme 3 on page 469) does not appear to involve a linkage of the type defined in formula (VI). For example, the only thiol group in product **7** of Soukchareun Scheme 3 is in the cysteine moiety, but it does not seem possible for that group to correspond to the thiol group in formula (VI). Additionally, the linking fragment -NH-C(O)-A-C(O)-NH- as required in the compound of claim 62 cannot be found in any of the Soukchareun compounds.

The Examiner further suggests that, according to the specification at page 2 of the present application, Soukchareun produces "the same" peptide-oligonucleotide conjugates as those presently claimed. Office action at page 3, first paragraph. The Examiner therefore concludes that Soukchareun anticipates the compounds of the presently claimed invention.

Applicants respectfully disagree. The description within the instant specification only notes that Soukchareun discloses a conjugation reaction between a maleimide-derived peptide and a 3'-cysteine functionalized oligonucleotide. This conjugation involves a reaction between the thiol group of a cysteine and a maleimide group tethered to a peptide (*See* Soukchareun page 467, col. 1, first full paragraph; and page 469, col. 2, first full paragraph). Such a reaction would not arrive at a compound of formula (VI), because the cysteine thiol group ends up as part of the linkage. Accordingly, the Soukchareun peptide-oligonucleotide conjugates would have the structure of Formula **A**.

[1] Although the Examiner refers to the compound of Formula (IV) in the Action (*See* page 2, last paragraph), applicants presume that he means a compound of Formula (VI). Acknowledgement of this error is respectfully requested.



(A)

The linkage of the oligonucleotide to the peptide through sulfur in Soukchareun is supported by the enclosed description of a reaction between a maleimide and a thiol (Exhibit A; obtained from www.piercenet.com). Further support is found in Xiao et al. (*Journal of Materials Science: Materials in Medicine* **1997**, 8, 867-872; Xiao; Exhibit B) wherein a cysteine moiety is coupled to a maleimide moiety (*See* Xiao, page 868, col. 2, last paragraph; and Fig 1) to form a linkage identical to that of Soukchareun (*See* Formula **A**).

The Examiner's conclusions may have resulted from a misreading of statements in the instant specification (e.g. on pages 18, lines 25-28 and page 32, line 11-14) that teach conjugation of maleimide peptides to some of the intermediates used to make the compounds of Formula (VI). Perhaps this has led the Examiner to conclude that identical products are obtained from Soukchareun and the process used to make formula (VI). That is not the case. It is respectfully submitted that the above remarks clarify the differences between the presently claimed compounds and those disclosed in Soukchareun.

In view of the foregoing remarks, it is believed that the Section 102 rejection as it applies to claims 62, 63, and 84-92 has been addressed and overcome.

III. Rejection Under 35 U.S.C. § 103

Claims 62, 63, and 84-92 have been rejected under 35 U.S.C. 103 as allegedly obvious over Soukchareun in view of Constancis et al. (US Patent No. 5,496,872, "Constancis"). The Examiner

asserts that it would have been obvious to use the Constancis linking structure (*See* Office Action, page 4) with the peptide residues and oligonucleotide residues of Soukchareun to arrive at the presently claimed compounds. According to the Examiner, the selection of the Constancis linker would merely constitute routine optimization and, thus, one of ordinary skill in the art would have a reasonable expectation of success in producing the presently claimed compounds.

In addition to the arguments pertaining to the Section 102 rejection, applicants further submit that the teachings of Soukchareun and Constancis, in view of one another, appear to be teachings in non-analogous art. Whereas the Soukchareun compounds are contemplated for use in biotechnology as peptide-oligonuleotide conjugates (*See* Soukchareun, abstract), the Constancis compounds lie in the field of adhesive compositions for surgical use (*See* e.g., col. 1, lines 4-7, col. 1, lines 49-52, and col. 11, lines 24-27). Additionally, Constancis is non-analogous art with regard to the peptide-oligonuleotide conjugates of the presently claimed invention and, thus, is not "reasonably pertinent to the particular problem with which the inventor was concerned" (*In re Oetiker*, 977 F.2d 1443, 1446, 24 USPQ2d 1443, 1445 (Fed. Cir. 1992)). Constancis would logically not "have commended [itself] to the inventor's attention in considering his problem" (*In re Clay*, 966 F.2d 656, 659, 23 USPQ2d 1058, 1060-61 (Fed. Cir. 1992)).

Because Soukchareun and Constancis lie in completely different technical fields, there would be no motivation for the skilled artisan to combine their teachings and arrive at the presently claimed compounds. The mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art suggests the desirability of the combination. *In re* Mills, 916 F.2d 680, 16 USPQ2d 1430 (Fed. Cir. 1990). The Examiner suggests that Constancis teaches "standard peptide residue linking," as though the linker used in the document was common general knowledge on the filing date of the present invention. However, the Examiner has provided no reason why a skilled artisan in the field of biotechnology conjugates would have been aware of the linker used in Constancis. Given that there is no reason to suppose that Constancis was common knowledge, then the skilled artisan would have needed a motivation to combine Constancis with Soukchareun. The Examiner has given no reason why the prior art suggests the desirability of such a combination.

Moreover, the Examiner states that Soukchareun teaches "the linking of peptide residue to an oligonucleotide under the like-kind standard chemical synthesis". However, it is not clear what "like-kind" of chemical synthesis the Examiner is referring to. As noted above, there are significant differences between the linking methodology disclosed in Soukchareun and that used to arrive at the compounds of Formula (VI) in the present application. Soukchareun must employ the aforementioned cysteine - maleimide coupling reaction and this method is substantially different from the cross-linking chemistry in Constancis. There is no coupling similarity between Soukchareun and either the present invention or Constancis. Furthermore, there is no teaching or suggestion in either Soukchareun or Constancis to remove the required cysteine - maleimide linker and replace it with the Constancis linker. Because the preparation of the Soukchareun and Constancis compounds involve entirely different chemistries, there would be no motivation to combine the documents to arrive at the presently claimed invention.

In light of the above arguments, the applicant respectfully submits that the Section 103 rejection as it applies to claims 62, 63, and 84-92 has been addressed and overcome.

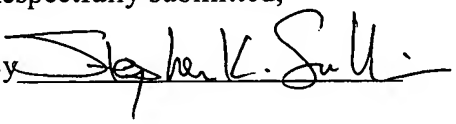
CONCLUSION

In view of the above remarks, this application is believed to be in condition for allowance, which is earnestly solicited.

If there are any other issues remaining which the Examiner believes could be resolved through either a Supplemental Response or an Examiner's Amendment, the Examiner is respectfully requested to contact the undersigned at the telephone number indicated below.

Dated: January 31, 2006

Respectfully submitted,

By 

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be generated by reduction of disulfide bonds. Alternatively, sulfhydryls can be introduced into
through reaction with primary amines using 2-Iminothiolane or Traut's Reagent (Product # 261
(Product # 26102) or SPDP (Product # 21857).

The maleimide group reacts specifically with sulfhydryl groups when the pH of the reaction mi
pH 6.5 and 7.5 and forms a stable thioether linkage that is not reversible (Figure 4). At neutral
react with sulfhydryls 1,000-fold faster than with amines, but at pH >8.5, the reaction favors pr
Maleimides do not react with tyrosines, histidines or methionines. Hydrolysis of maleimides to
maleamic acid can compete with thiol modification, especially above pH 8.0. Thiols must be e
reactions buffers used with maleimides because they will compete for coupling sites. Excess n
be quenched at the end of a reaction by adding free thiols. EDTA can be included in the coupl
minimize oxidation of sulfhydryls.

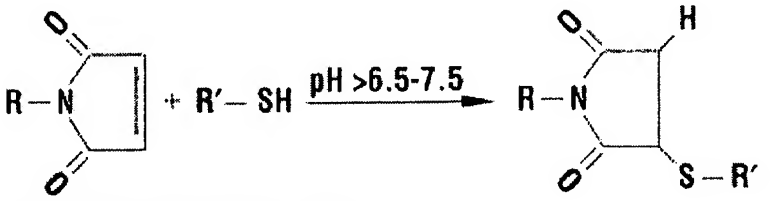

Figure 4. Maleimide reaction scheme.

Haloacetyls

The most commonly used α-haloacetyl cross-linkers contain the iodoacetyl group that react wi
groups at physiological pH. The reaction of the iodoacetyl group with a sulfhydryl proceeds by
substitution of iodine with a thiol producing a stable thioether linkage (Figure 5). Using a slight
iodoacetyl group over the number of sulfhydryl groups at pH 8.3 ensures sulfhydryl selectivity.
of free sulfhydryls, or if a large excess of iodoacetyl group is used, the iodoacetyl group can re
amino acids. Imidazoles can react with iodoacetyl groups at pH 6.9-7.0, but the incubation mu
longer than one week.

Histidyl side chains and amino groups react in the unprotonated form with iodoacetyl groups a
pH 7, respectively. To limit free iodine generation, which has the potential to react with tyrosin
tryptophan residues, perform iodoacetyl reactions and preparations in the dark. Avoid exposur
compounds to reducing agents. Available NHS-ester haloacetyl cross-linkers are listed in Tabl

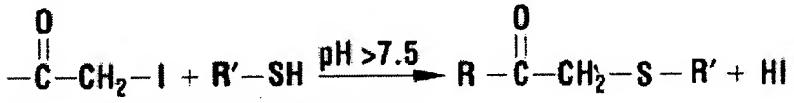

Figure 5. Active halogen reaction scheme.

Table 2. Available NHS-ester Haloacetyl Cross-linkers

Reagent	Reactivity	Product #
SIA	Amine/Sulfhydryl	22349
SIAB	Amine/Sulfhydryl	22329
Sulfo-SIAB	Amine/Sulfhydryl	22327

Pyridyl Disulfides

Pyridyl disulfides react with sulfhydryl groups over a broad pH range (optimal pH is 4-5) to forr
bonds, and therefore, conjugates prepared using these reagents are cleavable. During the rea
disulfide exchange occurs between the molecule's –SH group and the 2-pyridyldithiol group. A
pyridine-2-thione is released and its concentration can be determined by measuring the absor
nm (Figure 6). These reagents can be used as cross-linkers and to introduce sulfhydryl groups
The disulfide exchange can be performed at physiological pH, although the reaction rate is slo
3 for the pyridyldithiol cross-linkers available from Pierce.)

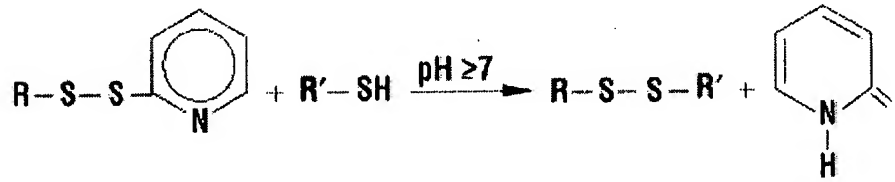

Figure 6. Pyridyl disulfide reaction scheme. Reaction efficiency can be monitored by deterr
concentration of the released pyridine-2-thione by measuring the absorbance at 343 nm (mola
coefficient at 343 nm = 8.08 x 10^3 M^{-1} cm^{-1}).

JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 8 (1997) 867–872

Immobilization of the cell-adhesive peptide Arg–Gly–Asp–Cys (RGDC) on titanium surfaces by covalent chemical attachment

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Surface modification of acid-pretreated titanium with 3-aminopropyltriethoxylsilane (APTES) in dry toluene resulted in covalently bonded siloxane films with surface coverage that was relatively controllable by regulating the reaction conditions. A hetero-bifunctional cross-linker, *N*-succinimidyl-3-maleimidopropionate (SMP), reacted with the terminal amino groups, forming the exposed maleimide groups. Finally, a model cell-binding peptide, Arg–Gly–Asp–Cys (RGDC), was immobilized on the surface through covalent addition of the cysteine thiol groups to the maleimide groups. X-ray photoelectron spectroscopy, radiolabelling techniques, and ellipsometry were used to quantify and characterize the modified surfaces.

1. Introduction

Titanium is a successful biocompatible material that is extensively used today for manufacturing bone-anchoring devices, such as dental implants or hip-joint fixation and replacement, as well as for pacemakers, heart-valves or ear-drum drainage tube coatings. It has advantageous bulk and surface properties: in particular, a low modulus of elasticity, a high strength-to-specific-weight ratio, excellent resistance to corrosion and a biocompatible surface oxide film [1]. The surface chemistry and structure are prime factors governing bone integration [2] and there is – from the view point of both surgeon and patient – considerable interest in increasing both speed of formation (healing time) and degree (long-term success) of close bone apposition for cement-free implantation.

Recent *in vitro* and *in vivo* studies on polymer and model oxide surfaces have demonstrated that specific surface–cell interactions and further cell organization can be mediated by surface-immobilized cell-adhesive peptides [3–7]. In our study we have immobilized the peptide Arg–Gly–Asp–Cys (RGDC) through silanization of a titanium metal surface, followed by reaction with a maleimide coupler – a technique adapted from procedures used for the covalent attachment of thiolipids to optical waveguides [8]. Fig. 1 shows the modification steps used in this work: first, silanization of a pretreated titanium surface (surface A) with 3-aminopropyltriethoxylsilane (APTES) in toluene, resulting in surface B with terminal amino groups; secondly, reaction of surface B with a hetero-bifunctional cross-linker, *N*-succinimidyl-3-maleimidopropionate (SMP), in *N*,*N*-dimethyl-formamide (DMF), resulting in surface C with exposed maleimide groups; finally, immobilization of a model cell-binding peptide Arg–Gly–Asp–Cys (RGDC) through covalent addition of the cysteine thiol (–SH) group to the maleimide group (surface D). This functionalization technique has a high flexibility, in the sense that it allows the attachment of any suitable biomolecule having a chemically accessible thiol group.

X-ray photoelectron spectroscopy (XPS), ^{14}C radiolabelling techniques, and ellipsometry were used to characterize and quantify the modified surfaces.

2. Materials and methods

2.1. Substrate and chemicals

Titanium coatings, 100 nm thick, on one side of commercial float glass samples (48 mm × 12 mm × 2 mm)

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Selected paper from the 13th European Conference on Biomaterials, Göteborg, Sweden.

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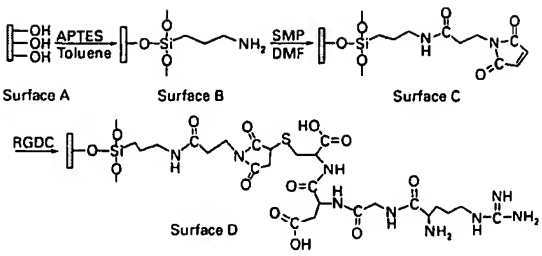

Figure 1 Sequence of the titanium surface modification procedure. Surface A corresponds to the pretreated titanium surface.

were produced in a Leybold Z600 DC-magnetron sputtering facility [9]. Pure water was obtained from an EASYpure™ device, Barnstead/USA. [^{14}C]-formaldehyde for radioactive labelling (specific activity 54 mCi mmol^{-1}) was purchased from Amersham, Buckinghamshire/UK. 3-aminopropyltriethoxylsilane (APTES) was obtained from Fluka, Buchs/Switzerland, distilled, stored, and used under a nitrogen atmosphere. RGDC was purchased from Bachem AG, Bubendorf/Switzerland, and all other chemicals from Fluka.

2.2. Surface analysis

XPS spectra were recorded using a Specs SAGE 100 system with unmonochromatized MgK_α radiation at 300 W (12 kV). Measurements were taken at a take-off angle of 90° with respect to the sample surface. The analysed area is typically 9×9 mm^2. Survey scans over a binding energy range of 0–1150 eV were taken for each sample with a constant detector pass energy range of 50 eV, followed by high-resolution XPS measurement (pass energy 14 eV) for quantitative measurements of binding energy and atomic concentration. Background subtraction, peak integration and fitting were carried out using SpecsLab software. Electron binding energies were calibrated to the hydrocarbon C 1s at 284.6 eV on pure titanium surfaces. To convert peak areas to surface concentration, sensitivity factors published by Evans *et al.* were used [10].

Surface film thickness was obtained using a Gaertner L-116C ellipsometer and software. The angle of incidence was set as 70°. The optical constants of the pretreated titanium surfaces were determined in at least four different areas on each individual substrate. The characterized substrates were then silanized. The thickness of the silanized organic film was calculated using the software for a single organic thin film and a refractive index of 1.44 [11].

2.3. Chemical functionalization

2.3.1. Pretreatment

Prior to silanization, the titanium-coated substrates were first pretreated by incubating them in a solution of 1 : 1 (vol/vol) methanol/HCl (37%) at room temperature for 30 min, followed by rinsing five times with water. The substrates were then treated in concentrated sulfuric acid at room temperature for 15 min, and again washed extensively with water. After treatment in boiling water for 10 min, they were washed five times with water, rinsed with acetone, and dried under vacuum for 12 h. The pretreatment resulted in a slight decrease of titanium coating thickness; XPS measurements, however, showed no glass to be exposed on the surfaces.

2.3.2. Silanization

Silanization was performed by incubating single substrate samples at 120 °C for 3 h in 30 ml dry toluene containing 0.5 ml (2.15 mmol) APTES. After reaction, the substrate was ultrasonically washed with chloroform (five times), acetone (twice), methanol (five times), and extensively with water. The APTES-modified substrates were stored in argon for XPS measurements and in water or organic solvents for further reactions as detailed below.

2.3.3. [^{14}C]-formaldehyde labelling of APTES-grafted surface

This was performed with the APTES-grafted samples mounted in a sample holder with four holes (diameter 7 mm), titanium metal surface facing upwards; 50 µl 10.0 mM $NaBH_3CN$ and 2.0 mM [^{14}C]-formaldehyde in CH_3CN were injected into each hole. After incubation for 12 h at 20 °C, the excess radioactive solution was removed and the exposed surface washed with CH_3CN ten times and water ten times. The sample was dried in a nitrogen jet and then cut into four pieces. The radioactivity of each piece was measured by scintillation counting in 5 ml scintillation fluid (1080 ml toluene, 920 ml Triton X-100, 5.4 g 2,5-diphenyloxazole, 0.2 g 1,4-*bis*-2-(5-phenyloxazolyl) benzene, and 40 ml acetic acid) on a Betamatic V liquid scintillation counter.

2.3.4. Preparation of maleimide-(surface C) and RGDC-grafted (surface D) substrates

A 24 mm × 12 mm silanized titanium substrate was placed in a small bottle with 400 µl *N*,*N*-dimethylformamide (DMF) containing 3 mg SMP (28 mM) with only the titanium surfaces contacting the solution. After incubation at room temperature for 1 h (with 2 min sonication every 10 min), the substrate was washed with DMF (five times) and water (ten times). To prevent hydrolysis, the substrates were immediately subject to the next chemical step: a 12 mm × 12 mm maleimide-grafted substrate was incubated at room temperature for 2 h (with 1 min sonication every 10 min) in 300 µl pure water containing 1.35 mg RGDC (10 mM). The pH had to be adjusted to 7 with 0.1 M NaOH because of the presence of CF_3COOH in the peptide solutions. The RGDC-grafted substrates were washed thoroughly with water, dried with nitrogen and stored in argon.

3. Results and discussion

3.1. Pretreated surface

The XPS data in Table I show that the pretreated surface A was relatively clean. The surface carbon concentration of 18% is typical for organic contaminant levels on "clean" oxide surfaces. No further contaminants were detected by XPS. Special precautions were taken to exclude silicon contamination (such as PDMS), which is a frequent problem as regards surface cleanliness and functionalization.

3.2. Silanization of the surface

The thickness of the organic siloxane layer and the number of reactive NH_2 groups introduced after silanization with APTES were quantified by XPS, ellipsometry, and [^{14}C]-formaldehyde radiology measurements (Table I). XPS survey spectra show signals of carbon, oxygen, titanium, nitrogen and silicon with intensities depending on the degree of silanization. After 3 h reflux silanization in dry toluene, only small signals of nitrogen and silicon in the range of 1%–3% surface concentration were detected, estimated to correspond to a submonolayer to monolayer coverage (approximately 0.5 nm from ellipsometric measurements). A surface coverage of 0.22 nmol NH_2-groups per cm^2 was calculated from radioactivity values of [^{14}C]-methylated surfaces [12]. Further incubation for another 12 h at room temperature (12 h RT in Table I) led to multilayer formation with a thickness of 2.6 nm and a surface coverage of 0.57 nmol NH_2-groups per cm^2, supported by the XPS results in Table I. If samples were washed after 3 h reflux silanization and returned to the same reaction solution for another 12 h at room temperature (wash + 12 h RT in Table I), a thicker siloxane film (ca. 5.2 nm) with a higher surface coverage of 1.54 nmol NH_2-groups per cm^2 was formed. Only a very weak titanium signal was detected by XPS in this case, confirming that the surface layer thickness is of the order of the XPS information depth (ca. 6 nm). The XPS N/Si atomic ratios in Table I show a gradual decrease with further increasing siloxane film thickness. This is likely to be the result of increasing lateral polymerization and network formation reducing the surface concentration of freely accessible amino groups.

3.3. Covalent attachment of peptide

For the further reactions of surface B with the crosslinker SMP and subsequently with RGDC, multilayer siloxane films (APTES 3 h reflux + 12 h RT) were chosen because both the attached siloxane and the underlying titanium can be obviously detected with XPS. The subsequent surface chemical changes were followed by XPS at each stage.

3.4. XPS chemical shifts and spectra deconvolution

The individual XPS spectra of C 1s, O 1s, and N 1s on surfaces A, B, C, and D are compared in Figs 2–4. Table II lists the proportion of the individual chemical states after deconvolution.

3.4.1. C 1s spectra

The major hydrocarbon peaks at 285.2 eV on surfaces B, C, and D (Fig. 2) appear at 0.6 eV higher energy compared to the hydrocarbon contaminants on the titanium metal surface A (284.6 eV). This is expected if the physically adsorbed hydrocarbons are replaced by the covalent-bonded APTES. The binding energy at 286.8 eV is assigned to amines (CH_2N) and alkoxy groups (CH_2–O). From surface B to C, an additional peak at much higher binding energy 289.0 eV attributed to carbon atoms in imide groups [C(=O)–N–C(=O)] strongly confirms the covalent bound maleimide groups. The increase at this position from C to D is attributed partly to carboxyl (*C*OOH) and guanidine [NH–C(=NH)–NH_2] carbons. C 1s energies of the amide functions (O=C–N) are expected at around 288.0 eV, but are difficult to be deconvoluted from the adjacent peaks. They contribute to both of the fitting peaks at 286.8 and 289.0 eV. The increase of intensity in the energy range 286.8–289.0 eV and the decrease at 285.2 eV from surface B to D reflect the results of the surface modification process.

3.4.2. O 1s spectra

Fig. 3 shows the evolution of O 1s peaks from surface A to B, C, and D. The main O 1s contribution in the case of titanium metal/oxide surface A is at a binding energy of 530.0 eV, typical for the natural TiO_2 surface layer [13]. The high-energy tail is attributed to surface hydroxides (and possibly organic contaminants). For surface B, a prominent siloxane O 1s peak at 532.5 eV is present; this peak increases with the siloxane surface coverage and is the only peak at high siloxane coverage of ca. 50 nm (not shown here). The O 1s position of the new amide functions (O=C–N) on surfaces C and D is expected at a position of 532.0 eV and overlaps with the siloxane signals. The carboxyl groups (*C*O*O*H) from RGDC sequence (surface D) cannot be separately deconvoluted and most likely also appear at around 532.0–532.5 eV. It is expected that the two carboxylic O 1s energies are not different if intraion or interion bonds are formed with amino moieties within the functionalized layer. By deconvolution of the two peaks at 530.0 and 532.5 eV (Table II), the intensity increase at 532.5 eV and decrease at 530.0 eV indicate that the surface chemistry changes from surface A to D.

3.4.3. N 1s spectra

Surface B shows two peaks at 399.7 eV (75%) (free amino groups) and 401.7 eV (25%, H-bonded and/or positively charged amines), also observed by other groups for APTES-modified titanium surfaces [14]. On surface C the imide [C(=O)–N–C(=O)] (N 1s at 400.6 eV) is introduced and part of the amine functions on surface B are converted to amides (O=C–N, N 1s at 400.0 eV). This can explain the shift of the peak

TABLE I Pretreated and APTES silanized titanium surfaces characterized with XPS for elemental composition, ellipsometry for surface layer thickness, and radioactivity measurements for surface coverage. The standard deviations refer to five samples from different batches

Sample	Elemental composition						Thickness (nm ± S.D.)	Radio activity (nCi cm^{-2} ± S.D.)	Surface coverage[b] (nmol cm^{-2} ± S.D.)
	C (% ± S.D.)	O (% ± S.D.)	Ti (% ± S.D.)	N (% ± S.D.)	Si (% ± S.D.)	N/Si[a]			
Pretreated titanium	18.3 ± 1.4	58.4 ± 2.6	23.3 ± 1.3						
APTES 3 h reflux	18.5 ± 1.3	54.0 ± 2.3	22.7 ± 1.7	2.2 ± 0.8	2.6 ± 0.8	0.85	0.4 ± 0.2	11.7 ± 3.1	0.22 ± 0.06
APTES 3 h reflux + 12 h RT	24.3 ± 2.8	50.0 ± 3.4	16.1 ± 2.2	4.1 ± 1.3	5.4 ± 1.3	0.76	2.6 ± 0.4	30.4 ± 4.6	0.57 ± 0.09
APTES 3 h reflux + wash + 12 h RT	48.4 ± 4.6	28.2 ± 2.7	1.5 ± 0.8	8.7 ± 1.9	13.3 ± 2.7	0.66	5.2 ± 0.6	81.9 ± 11.7	1.54 ± 0.22

[a]Mean atomic ratio of N/Si.
[b]Assuming a molar ratio [H"CHO]/[NH_2] = 1, refer to [12].

Figure 2 XPS O 1s spectra of surfaces A, B, C, and D.

Figure 3 XPS C 1s spectra of surfaces A, B, C, and D.

Figure 4 XPS N 1s spectra of surfaces B, C, and D.

maximum to 400.6 eV. On surface D, the increase of high binding energy peaks at 401.7 eV is clearly due to the more positively charged nitrogen species in guanidine and amino moieties forming intraion or interion bonds with carboxyl groups.

3.5. Estimation of reaction yields

Because of the inevitable carbon contamination and oxygen concentration on the starting surface A,

TABLE II Results of deconvolution of the XPS spectra in Figs 2, 3, and 4: binding energies (eV) and relative (Σ = 100%) intensities of the deconvolution peaks

Surface	C 1s			O 1s		N 1s		N/Si[b]
	285.2 eV	286.8 eV	289.0 eV	530.0 eV	532.5 eV	399.7 eV	401.7 eV	
A	71.1%[a]	21.2%[a]	7.7%[a]	87.3%	12.7%	–	–	–
B	68.1%	27.2%	4.7%	62.2%	37.8%	75.2%	24.8%	0.70
C	57.1%	31.3%	11.7%	55.0%	45.0%	61.4%	38.6%	0.89
D	50.3%	34.5%	15.2%	51.2%	48.8%	44.2%	55.8%	1.13

[a]The C 1s binding energies of the pretreated titanium surface A are 284.6, 286.1, and 288.5 eV.
[b]Mean atomic ratio of N/Si.

quantitative statements as regards reaction yields for the subsequent modification reactions, are difficult or impossible. The N 1s signal has been used by Ratner as a quantitative measure of the amount of proteins on a surface [15]. Here the N 1s signals are used to calculate the reaction yields. However, the absolute atomic concentrations depend on the measured volume and the chemical components in this volume. Changes of the chemical components and the film thickness on surfaces B, C, and D make the estimation difficult. Fortunately, silicon, which is not present on the starting surface A, can be used as an internal reference. The absolute silicon surface concentration remains constant on surfaces B, C, and D (no hydrolytic decomposition of the siloxane layer during the subsequent modification steps was detected by XPS). Because the whole modified layer thickness is below the information depth of XPS, the reaction yield can be deduced from the following simple formula

Reaction yield (B → C) =

$$\frac{\text{(number of imide-N on surface C)}}{\text{(number of N on surface B)}} = \frac{n_{N(C)} - n_{N(B)}}{n_{N(B)}} = \frac{(N/Si)_C - (N/Si)_B}{(N/Si)_B}$$

$$= \frac{0.89 - 0.70}{0.70} = 0.27 \qquad (1)$$

Reaction yield (C → D) =

$$\frac{\text{[(number of N in RGDC)/7]}}{\text{(number of imide-N)}} = \frac{[n_{N(D)} - n_{N(C)}]/7}{n_{N(C)} - n_{n(B)}} = \frac{[(N/Si)_D - (N/Si)_C]/7}{(N/Si)_C - (N/Si)_B}$$

$$= \frac{(1.13 - 0.89)/7}{0.89 - 0.70} = 0.18 \qquad (2)$$

where $n_{N(i)}$ is the absolute nitrogen atomic concentration of surface i, and $(N/Si)_i$ is the atomic ratio of nitrogen to silicon of surface i from Table II. The divisor 7 takes account of the seven nitrogen atoms in one RGDC molecule. The conclusion from the semiquantitative coverage estimations is that under the chosen experimental conditions, only about one-fourth of amines on surface B were converted to maleimide functions and one-fifth of maleimides converted to a RGDC-grafted surface. The optimum reaction conditions are still under investigation. Combining the NH_2 surface coverage of 0.57 nmol cm^{-2} (Table I) and the reaction yields, the peptide surface coverage was calculated to be approximately 0.03 nmol cm^{-2}. This corresponds to submonolayer surface coverage regardless of the "lying-down" conformation of peptides. However, it is not likely that a full surface coverage of peptides is optimum for specific cell recognition and the currently achieved coverage can be expected to be sufficiently high to influence cell-surface adhesion [3].

4. Conclusion

The present results show that the model cell-adhesive peptide, Arg–Gly–Asp–Cys (RGDC), can be covalently bound to titanium (oxide) surfaces by the described procedure using silanization, cross-linking, and peptide attachment through the cysteine thiol group. The individual reaction steps were semiquantitatively characterized by X-ray photoelectron spectroscopy (XPS), radiolabelling techniques, and ellipsometry. The RGDC surface coverage is estimated to be 0.03 nmol cm^{-2}, expected to be high enough for specific surface–cell interactions. Future perspectives of this study include the investigation of protein adsorption and osteoblast interaction with peptide-functionalized titanium surfaces.

Acknowledgements

The authors thank Dr. P. Böni of Paul Scharrer Institut, CH 5232 Villingen PSI, Dr H. Chai-Gao and M. O. Bucher, CSEM, Dr P.-H. Vallotton, Institut Straumann, CH-4437 Waldenburg, and M. Windler, Sulzer Orthopaedics, CH-8404 Winterthur, for their support. This study was financed by the Swiss Priority Program of Materials (PPM).

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Received 12 May
and accepted 12 May 1995